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14. ABSTRACT

Most rheumatoid arthritis (RA) patients rely on glucocorticoids (GCs) at some point during the disease. GCs signal through the GC receptor (GR), a transcription factor that in addition to binding DNA directly can 'tether' to DNA-bound AP1 and NFkB and repress their numerous pro-inflammatory target genes. We discovered that GR-Interacting Protein (GRIP)1 in macrophages (MΦ) serves as a novel GR corepressor. Notably, GR:GRIP1 complexes repress pro-inflammatory genes of *two classes*: those activated through RNA polymerase (Pol)II recruitment and transcription initiation; and others, pre-loaded with paused Pol II that requires a signal for entry into productive elongation. We aim to dissect the role of MΦ GRIP1 as a driver of anti-inflammatory actions of GCs at the level of GR transcription complexes at genes of each regulatory class and in mouse models of RA. Having established technologies to identify GR:GRIP1-regulated genes in MΦ genome-wide, we are creating cistromes of where GR, GRIP1 and Pol II bind in inflammatory and GC-treated MΦ. We have made a substantial progress in understanding mechanistically how GR:GRIP1 repress pro-inflammatory genes of different classes. Using representative genes of each class, we are able to dissect the differences in step-wise assembly of the activation complexes that become targets for GR repression.

15. SUBJECT TERMS

Rheumatoid arthritis, inflammation and autoimmunity, macrophages, glucocorticoid receptor, transcriptional regulation, coactivators and corepressors, cytokines, mouse models of disease

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1. INTRODUCTION:

Up to 80% of rheumatoid arthritis (RA) patients rely on glucocorticoids (GCs) at some point during the disease however, serious side effects of traditional GCs demand a better understanding of their actions so that the anti-inflammatory and adverse effects can be uncoupled. GCs signal through the GC receptor (GR), a ligand-dependent transcription factor that recruits numerous cofactors into target gene-specific regulatory complexes to activate or repress gene expression (2). Many of the immunosuppressive properties of GCs stem from the ability of GR to 'tether' to DNA-bound activator protein-1 (AP1) and nuclear factor-kB (NFkB) and repress their targets. We discovered that GR-Interacting Protein (GRIP)1 in macrophages (MΦ) – a central cell type in RA pathogenesis – serves as a novel GR corepressor (3). Notably, GR:GRIP1 complexes repress inflammatory cytokine genes of *two distinct classes*: those activated through RNA polymerase-II (Pol II) recruitment and transcription initiation; and others, pre-loaded with PoII that initiates transcription but 'stalls' in the promoter-proximal region bound by the Negative Elongation Factor (NELF), and requires a signal for NELF release and entry into productive elongation (1,4). Our objective is to dissect the role of GRIP1 as a driver of anti-inflammatory actions of GCs in MΦ at the level of GR transcription complexes at genes of distinct regulatory classes and in mouse models of RA *in vivo*. The completion of this project will provide a new insight into the genome-wide GC regulation of inflammatory genes and reveal the utility of GRIP1 as a potential predictive marker for RA severity and effectiveness of GC therapy. These studies will foster the design of 'new generation' GR:GRIP1-targeting drugs that suppress inflammation while bypassing adverse side effects – a goal relevant not only to RA but to Paget's disease, scleroderma, psoriasis and other autoimmune disorders.

2. KEYWORDS:

Rheumatoid arthritis, glucocorticoid receptor, transcriptional regulation, gene repression, inflammation and autoimmunity, macrophages, coactivators and corepressors, RNA polymerase initiation and elongation, genome-wide analysis.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Task 1. Identify in MΦ transcription initiation- *vs.* elongation-repressed GR:GRIP1 target genes relevant to RA pathogenesis (mos 1-24)

1.1 Identify the genome-wide distribution of GR, GRIP1, PolII and NELF in MΦ under inflammatory and anti-inflammatory conditions (mos 1-24).
These studies will proceed immediately upon project initiation; the optimization of anti-GRIP1 antibodies is currently ongoing. We are also optimizing the conditions of sonication for the best performance and yields in ChIP-Seq (as they appear to differ from those we routinely use for ChIP-PCR). The computational platform is fully set-up for data processing.

1.2 Assess the role of GRIP1 to GR-mediated repression of inflammatory genes in MΦ (mos 12-30).
We anticipate to start RNA-Seq experiments by the beginning of year 2, when the first round of ChIP-Seq experiments has been completed and data processed. We expect to complete expression profiling by the end of year 2 or mid year 3.

Task 2. Determine the molecular mechanisms of GR:GRIP1-mediated repression of inflammatory genes (mos 1-36)

Because we already have a set of candidate genes of both classes for these studies we can initiate the in-depth mechanistic studies on some of them immediately without waiting until genome-wide

approaches described in Aim 1 are completed. For both Sub-Aims, the rate-limiting step will be maintaining the ChIP-sufficient number of GRIP1 KD mice. So the experiments will be planned in a way that only one subset of genes at a time requires these mice of the analysis of candidate cofactors.

2.1 Dissect the composition and function of GR:GRIP1 repression complexes at RA-relevant *initiation-controlled genes* (mos 1-36).

2.2 Identify the mechanisms of GR:GRIP1-dependent repression of pro-inflammatory *elongation-controlled genes* (mos 1-36).
In addition to the general layout of experiments described above for this Task, the ChIP-Seq analysis of NELF binding in GRIP1-KD mice will likely be carried out at late stage of the project, year 3 should this approach appear promising.

Task 3. Investigate the role of GRIP1 *in vivo* in mouse models of arthritis (mos 7-33)

3.1 Assess the role of GRIP1 *in vivo* using the K/BxN serum-induced arthritis model (mos 7-18). This is the first straightforward mouse model of RA we plan to test, for which will start breeding mice immediately, begin the experiment mid-year-1 and plan to complete by mid-year-2.

3.2 Evaluate the role of GRIP1 *in vivo* in the collagen-induced arthritis (CIA) model (mos 19-30). CIA model will follow the K/BxN model; we anticipate a year for these experiments'

3.3 Assess GRIP1 expression over the course of the disease in K/BxN and CIA models (mos 13-33).

What was accomplished under these goals?

Task 1.
We have made a significant progress under proposed Aim1. At this point, we have completed RNA Pol II ChIP-seq in BMMΦ (1 replicate with all 4 treatment conditions con/Dex/LPS/LPS+Dex and 2 replicates with con/LPS). A preliminary analysis of these data suggested that of about 150 genes that were repressed by GCs in MΦ, approximately half were controlled at the level of Pol II recruitment and transcription initiation, and about 1/3rd were occupied by stalled Pol II prior to induction with LPS signal triggering pause release and productive elongation, and Dex re-instating the pause (**Fig. 1**). Interestingly, we noticed a few genes, which were activated and repressed at distinct steps of the transcription cycle, e.g., when activation occurred by Pol II recruitment, but GR promoted Pol II pausing, and, conversely, when the gene was stalled prior to induction, but GR-mediated repression triggered loss of Pol II from the gene including TSS (**Fig. 1**). These findings bring up an important possibility that some genes that have been repressed by GR previously may acquire a novel transcriptional state and respond differently to subsequent stimulation.

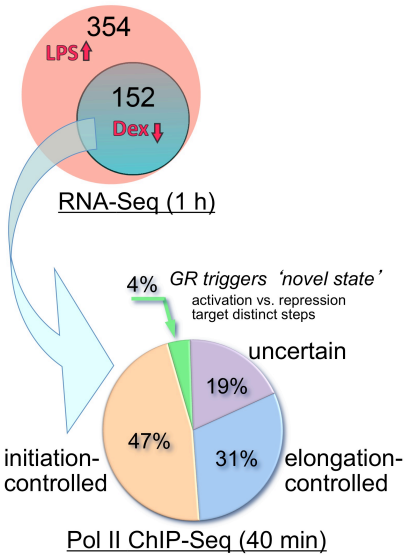

Fig. 1. RNA-seq dataset (1) shows 354 genes upregulated by LPS >2x and 152 genes repressed by LPS+Dex >1.4x in BMMΦ. Pol II ChIP-seq profiles of 152 repressed genes were examined for the presence of a defined peak in the vicinity of TSS in untreated, Pol II distribution throughout the gene body in LPS-treated, and a concentration of the TSS-associated Pol II peak in LPS+Dex-treated MΦ to achieve a preliminary assignment to initiation- or elongation-controlled class. Genes with a 'weak' pause, multiple TSS, low Pol II occupancy, or overlap with other genes were temporarily unassigned. Genes that demonstrated a paused configuration before activation but not in a repressed state or vice versa we deemed to acquire a 'novel state'.

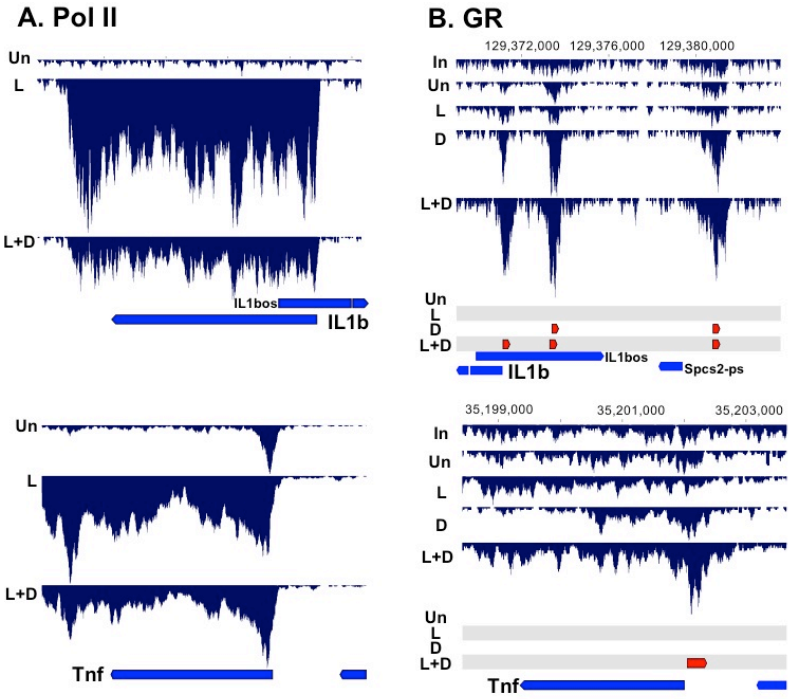

Fig. 2. Analysis of Pol II and GR occupancy by ChIP-seq. BMMΦ were untreated (Un) or treated with 10 ng/ml LPS (L), 100 nM Dex (D) or both together (L+D) for 45 min and ChIP-seq was performed using Pol II (**A**) or GR (**B**) polyclonal abs. Read density profiles for Il1b (top) and Tnf (bottom) were constructed by aggregating individual reads over 100-bp windows. Read distribution in inputs (In) was used to establish background. Peaks were called (marked in red) using CLC Genomics Workbench V 7.5. (Qiagen).

While the global analysis of Pol II cistromes is ongoing, the Pol II ChIP-seq tracks of the two stereotypic representatives of the 'initiation-controlled' and 'elongation-controlled' groups of genes are shown in **Fig. 2A**. In the case of Il1b, there is no appreciable Pol II occupancy across the gene prior to stimulation, there is a striking increase in Pol II at the TSS and the body of the gene in response to LPS, and this occupancy is dramatically reduced in the presence of Dex (**Fig. 2A, top**). In the case of Tnf, Pol II initiates transcription and stalls at the TSS prior to stimulation, LPS treatment releases the pause increasing Pol II density at the TSS as well as throughout the body of the gene and Dex greatly attenuates pause release resulting in the persistence of the Pol II peak near the TSS (**Fig. 2A, bottom**).

Because the project relies heavily on ChIP-seq, we put a lot of effort in protocol optimization. In the course of these studies, we switched to double-crosslinking protocols that use disuccinimidyl glutarate (DSG) followed by formaldehyde, which dramatically improved signals, especially for difficult-to-ChIP proteins, such as GR at 'tethering' sites and cofactors that do not bind DNA directly. We then assessed GR binding to chromatin in two biological replicates of GR ChIP-seq in untreated, Dex-, LPS- and LPS+Dex-treated BMMΦ. GR binding to chromatin in the absence of Dex was minimal (con - 295 peaks, LPS alone - 332 peaks $p<0.01$) whereas, 12,218 and 6,542 peaks ($p<0.01$) were detected following a 45 min Dex and LPS+Dex treatments, respectively.

With respect to the representative Il1b and Tnf loci bearing NFkB sites/'tethering GREs' at -158 bp, -2.3 kb, -10 kb and 220 bp, respectively, at which have previously described binding of GR, p65 and GRIP1 (3), GR ChIP-seq revealed little occupancy in untreated or LPS-treated BMMΦ. Dex alone triggered some binding at the Il1b -2.3 kb and -10 kb NFkB sites, but it was significantly enhanced in LPS+Dex co-treated BMMΦ (**Fig. 2B, top**). Furthermore, the proximal NFkB sites at Il1b -158 bp and at Tnf -220 bp gain statistically supported GR peaks only in LPS+Dex co-treated BMMΦ (**Fig. 2B**). Thus, our GR ChIP-seq in BMMΦ was efficient enough to detect GR bound at established tethering sites and corroborated the requirement for both LPS and Dex co-treatment for efficient GR recruitment.

Task 2.

Experiments proposed under Aim 2 are aimed at dissecting the molecular mechanisms underlying GR:GRIP1-mediated repression for initiation- and elongation-controlled genes. Thus far, we have made the biggest progress in our understanding of how *initiation-controlled* genes are repressed and, therefore this part of the project is described in the current Progress Report. Our ChIP assays (3) have demonstrated that increased Pol II recruitment in response to LPS at such genes (also shown by ChIP-seq in **Fig. 2A** for Il1b) is blocked by Dex; this correlated with an increase in histone H3 (3) and H4 (**Fig. 3**) acetylation, which was similarly attenuated by Dex. Importantly, these changes in acetylation were also limited to initiation-controlled genes, and were not seen at elongations-controlled Tnf (**Fig. 3**). Modifications are read by so-called histone code 'readers' that then convey signaling information to additional coregulators and basal transcriptional machinery. We focused on a family of the bromodomain and extra-terminal containing Bet proteins (Brd2, 3 and 4) which bind AcH3 and AcH4 and facilitate the formation of transcription complexes (5). Importantly, small-molecule Bet inhibitors (I-Bet) that bind Bet domains thereby competitively inhibiting their association with acetylated histones are commercially available (6,7). We hypothesized that if recruitment of Brd proteins is important for activation of a specific subclass of pro-inflammatory genes, I-Bets will inhibit their activation. **Fig. 4** demonstrates that IBET, in a dose-dependent manner, inhibited LPS-induced induction of Il1b but not Tnf, consistent with the potential role of Brd proteins in activating specifically initiation-controlled genes.

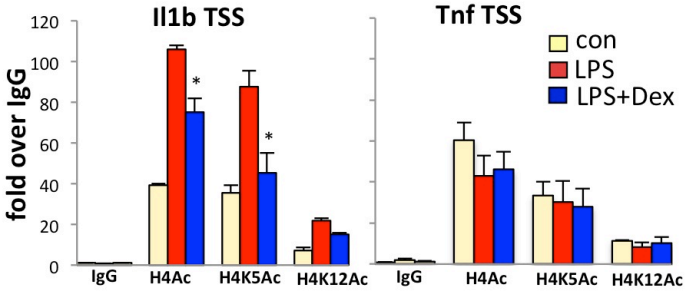

Fig. 3. Analysis of histone H4 acetylation at GR-sensitive genes. BMMΦ were treated with 10 ng/ml LPS±100 nM Dex for 30 min and histone H4 Pan/K5/K12Ac at the TSS of indicated genes was assessed by ChIP. For each location, qPCR signals were normalized to those at control r28S gene and expressed as relative enrichment over normal IgG (=1). Shown are mean ± SEM (n=3). * p< 0.05, calculated using two tailed t-Student test.

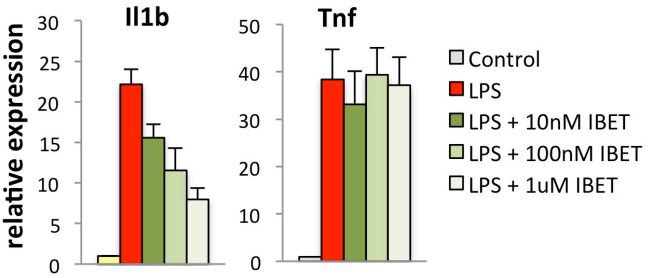

Fig. 4. IBET specifically inhibits LPS-induced expression of IL1b in a dose dependent manner. BMMΦ were treated with 10 ng/ml LPS± BET for 30 min and the levels of indicated transcripts were assayed by RT-qPCR, normalized to bActin and expressed relative to those in untreated MΦ (=1). Shown are mean ± SEM (n=4). * p < 0.05, calculated using two tailed t-Student test.

The selectivity of IBET actions suggested the LPS-induced histone acetylation and, importantly, its sensitivity to Dex may lead to similarly selective utilization of the Brd proteins at a specific gene class. We chose to focus on Brd4 because this family member has been previously shown to interact with subunits of the Mediator complex and contribute to the regulation of pro-inflammatory NFkB target genes. Hence, we evaluated the occupancy of Brd4 at Il1b and Tnf following LPS or LPS+Dex treatment. Consistent with the histone acetylation and IBET data, we observed Brd4 recruitment to Il1b in response to LPS and its inhibition by Dex; conversely, Brd occupancy at the Tnf TSS was constitutive (**Fig. 5**). Brd4 has been shown to interact with numerous transcriptional regulatory proteins including transcription factors, e.g. NFkB (8), cofactors and components of basal transcriptional machinery. In particular, several studies

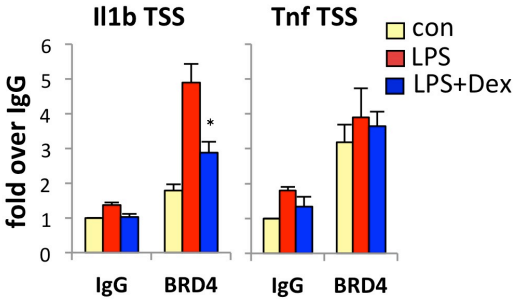

Fig. 5. GR inhibits BRD4 recruitment to initiation controlled Il1b. BMMΦ were treated with 10 ng/ml LPS±100 nM Dex for 30 min and BRD4 occupancy at the TSS of indicated genes was assessed by ChIP-qPCR exactly as in Fig. 3.

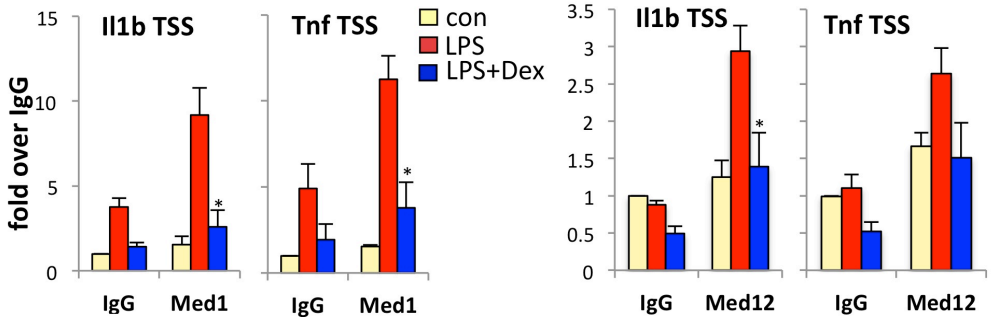

Fig. 6. GR inhibits Med1 and Med12 recruitment to both classes of genes. BMMΦ were treated with 10 ng/ml LPS±100 nM Dex for 30 min and Med1 and Med12 occupancy at the TSS of indicated genes was assessed by ChIP-qPCR as described in Fig. 3.

showed that Brd4 interacts with the Mediator complex, and specifically with Med1 and Med12 subunits (9). Because Mediator is implicated in facilitating the recruitment Pol II to target promoters, we questioned whether by inhibiting histone acetylation and the recruitment of Brd4, GR disrupts loading of the Mediator. **Fig. 6** shows that the recruitment of both Med1 and Med12 was stimulated by LPS in a Dex-sensitive manner. Interestingly this was observed at both Il1b and Tnf TSS, suggesting that GR may also inhibit Mediator loading using more than one mechanism.

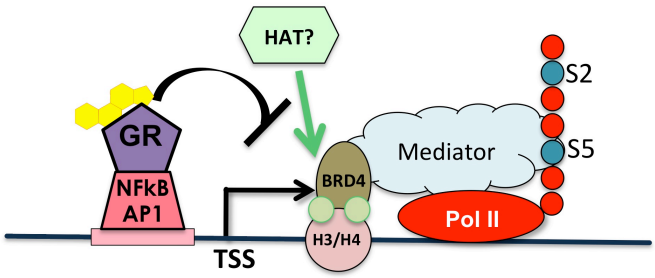

Fig. 7. A model for GR-mediated repression of initiation controlled genes.

Collectively, our data thus far are consistent with a model whereby at initiation-controlled genes, such as Il1b, GR inhibits histone H3 and H4 acetylation, leading to reduced recruitment of Brd4, the Mediator complex and ultimately Pol II, thereby attenuating transcription initiation (**Fig. 7**).

Task 3.

Aim 3 investigates the implications of GR:GRIP1-mediated repression of inflammatory genes in MΦ using mouse models of inflammatory arthritis.

We have completed the study with KBxN model of arthritis in WT and conditional GRIP1 KO (cKO) mice lacking GRIP1 in MΦ. We found this model to be not sufficiently informative for testing our hypothesis on the loss of protective role of MΦ GRIP1 in RA. Although histological analysis revealed a slight increase in the roughening of the synovial membrane and in the number of adhering cells in GRIP1 cKO mice (**Fig. 8B; arrow**), the differences were too subtle to pursue, and based on the ankle thickness and clinical disease scores, the severity of arthritis was similar in mice of both genotypes (**Fig. 8A**). Indeed, this is an acute model with a rapid onset and resolution, in which WT animals develop maximal disease making it difficult to assess arthritis exacerbation in the cKO.

Gene expression analysis from total RNA from ankles did reveal a modestly higher level of the Il1b transcript in GRIP1 cKO (data not shown), however, the other cytokine that is critical in human RA pathogenesis and, in our studies, undergoes derepression in cKO MΦ is Tnf; unfortunately, the KBxN model is TNF-independent and, indeed, we observed no TNF induction in inflamed arthritic ankles relative to unaffected ones, making any comparison between genotypes uninformative. Overall, the acute, TNF-independent disease progression and resolution in the KBxN model, being very different from human disease, suggested that this system will not be useful for studying the protective role of MΦ GRIP1.

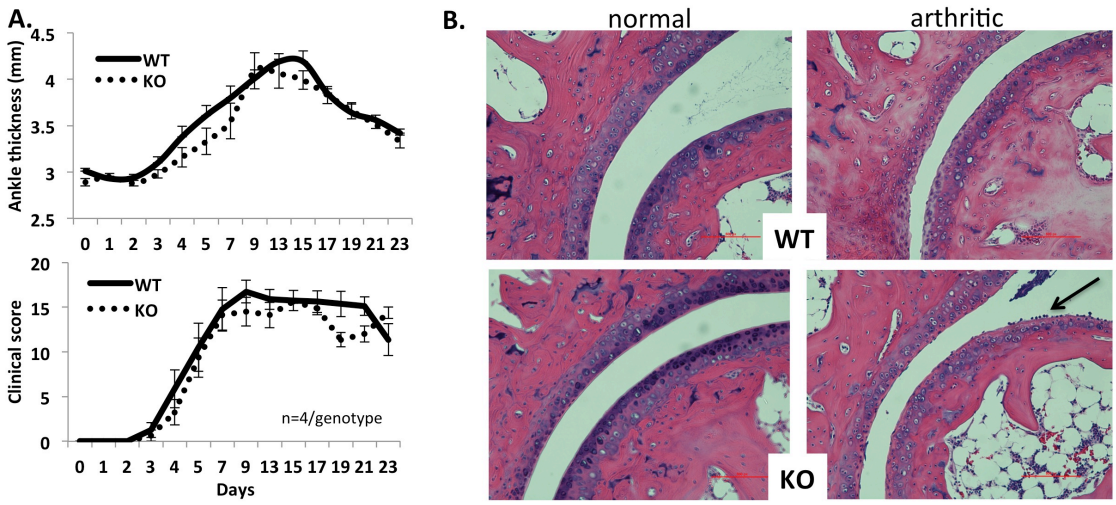

Fig. 8. Development of KBxN arthritis in WT and conditional GRIP1 KO mice. A. Ankle thickness and clinical score of control (n=4) and GRIP1 KO (n=4) mice injected with KBxN serum (mean±SD) was monitored for over 23 days. Mice were injected on day 0 and day 2. A caliper was used to measure ankle thickness. Clinical scores shown represent average per paw where maximum score per paw is 20. **B.** Histology of affected joints in WT and GRIP1 KO mice at day 14.

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What opportunities for training and professional development has the project provided?

This project has provided extensive training opportunities for several individuals in the Rogatsky lab.

The main contributor to the project is **Maria Sacta**, an MD-PhD student in the lab; a large part of the project is her thesis work. Ms. Sacta has made outstanding progress, presented her work at multiple research-in-progress seminar series on campus, and, in the current reporting period, at the Cold Spring Harbor Nuclear Receptors & Disease meeting (poster; October 2014), and EMBO Nuclear Receptors Conference in Corsica, France (poster; September 2015). Ms. Sacta is also first co-author with Dr. Chinenov (see below) of a prestigious review in Annual Reviews in Physiology (http://www.ncbi.nlm.nih.gov/pubmed/26667074). Ms. Sacta is beginning to assemble her main first first-author manuscript entirely on the subject of this DOD prohect.

Dr. Yurii Chinenov is responsible for the computational part of the project, especially as related to genome-wide analyses. He has gained a very significant experience during the reporting period and has become the official 'go-to' person for the HSS research community when help with bioinformatics is needed. He has co-organized several Genomics Workshops (2015 Epiworkshop co-organized by the Weill Cornell Medical College, HSS and New York City College of Technology; 2015 Chip-Seq data analysis University of South Carolina Bioinformatics and OMICS group; 2015 The Hospital for Special Surgery workshop on genomics data analysis) and has been appointed as a co-investigator of the HSS Genomics Center.

Dr. Maddalena Coppo is a Postdoctoral Fellow who is handling her independent project. However, she is an extremely talented experimentalist and the first one in the lab to firmly introduce the ChIP-seq technology into our line of techniques. The Pol II and GR ChIP-seq datasets were generated by Dr. Coppo through a painstaking process of assay optimization that took many months. During the reporting period, Dr. Coppo presented her work at several research-in-progress series on campus and at the Cold Spring Harbor Nuclear Receptors & Disease meeting (poster; October 2014), she attended Genomics workshops offered on campus, co-authored a review in Molecular Endocrinology (see 'Products') and has two research manuscripts currently in review.

Dr. Inez Rogatsky the PI on the project, has presented work at three conferences and two seminars (10/2014 - Cold Spring Harbor Meeting. Nuclear Receptors & Disease, Cold Spring Harbor NY; 04/2015 - Seminar. Rutgers, The State University of New Jersey, Newark, NJ; 05/2015 - Seminar. University College London, United Kingdom; 08/2015 - FASEB Conference. Molecular and Systems Integration of Genomic and Nongenomic Steroid Hormone Action. Big Sky, MT; 09/2015 - EMBO Conference. Nuclear receptors: From Molecules to Humans. Ajaccio, France), reviewed manuscripts for multiple journals, has served as an ad hoc reviewer for the NIH MCE Study Section in September-October 2014, and for the NIH Special Emphasis Panel in October-November 2015. At the end of the reporting period Dr. Rogatsky was being evaluated for a promotion to the rank of Full Professor (Weill Cornell Medical College) and a Senior Scientist (HSS). Publications are listed under 'Products'.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Under Task 1:

A significant amount of work is currently ongoing to carry out GRIP1 ChIP-seq and NELF ChIP-seq experiments in BMMΦ. In addition, we have been able to obtain a conditional NELF-E KO mice

lacking functional complex in myeloid cells. Using these systems, we will assess a requirement for NELF for GR-mediated repression of inflammatory genes on a genome-wide scale. In addition, we will classify all GR-repressed target genes into distinct transcriptional groups and begin to identify the molecular features that dictate their specific mechanism of regulation. We will quantify, for example, whether GRIP1 co-repressor function is more prevalent in a specific class of GR targets. Importantly, we will be able to document the rare examples of genes at which GR appears to switch the rate-limiting step of their regulation and assess how long-lived this novel transcriptional state is.

Under Task 2:
For elongation-controlled genes, we will probe for a physical interaction between GR:GRIP1 and any of the NELF subunits. We will assess whether NELF retention at the TSS of repressed genes is solely the consequence of a block in the recruitment of CDK9 or if there is a more direct impact of GR:GRIP1 on NELF.
With respect to initiation-controlled genes, we plan to evaluate potential H3/H4 HATs for their ability to rescue the induction of inflammatory genes despite the presence of GCs. This will be done using overexpression systems. At the same time, we will evaluate the recruitment of potential endogenous HATs to GR-sensitive genes in response to gene activation and the impact of Dex on this recruitment. Our goal here is to identify the H3/H4 HAT whose occupancy is potentially attenuated by GR, leading to reduced HS/H4 acetylation, loss of Brd4, Mediator and ultimately block in Pol II recruitment.

Under Task 3:
Because of the outcome of the KBxN model with respect to the role of GRIP1 in inflammatory arthritis was uninformative, we will assess the effect of conditional GRIP1 deletion in the collagen-induced arthritis model. This model is not highly penetrant in the WT C57B/6 mice; because GRIP1 deletion is predicted to exacerbate the disease, we may expect GRIP1 KO to display a more dramatic phenotype relative to WT.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Rheumatoid Arthritis (RA) is an autoimmune disease of the joints that affects 1.5 million adults in the US. Chronic inflammation in RA is driven by immune cells such as macrophages that migrate into the affected joints and produce small proteins called inflammatory cytokines. This proposal aims to understand how glucocorticoids (GCs) – an extremely commonly used type of drugs in RA – affect macrophage function. GCs work through the GC receptor (GR), a protein which binds DNA and directly turns many target genes on (activation) and off (repression) including those that encode inflammatory cytokines. GR does not work in isolation, and we recently described a GR accessory protein GRIP1 which works as a 'corepressor' helping GR to turn off inflammatory genes. Interestingly these genes fall into two different groups (classes) based on how inflammatory signals activate them. How these genes are repressed by GR and what is the role of GRIP1 in this process is unknown. Our overall goal is to understand how GR:GRIP1 complexes repress inflammatory genes in macrophages in cell culture and in mouse models of RA.

During the past year, we have established the cutting edge technology to begin identifying GR:GRIP1-regulated genes in macrophages genome-wide. We are in the process of creating the 'cistromes' or genome-wide maps of where GR, GRIP1 and RNA Polymerase II - the enzyme that is responsible for transcribing DNA to RNA - bind in inflammatory macrophages or those that have been treated with GCs. Having these maps will simplify the task of understanding mechanistically at a molecular level how GR:GRIP1 repress pro-inflammatory genes of different classes. Through our comparative analysis of representative genes of each class, we are able to dissect the differences in step-wise assembly of the protein complexes that turn them on and that become targets for repression by GR upon GC treatment.

What was the impact on other disciplines?
"Nothing to Report."

What was the impact on technology transfer?
"Nothing to Report."

What was the impact on society beyond science and technology?
Ms. Maria Sacta, the graduate student whose PhD thesis project is centered largely on this funded DOD award, is identified as a 'minority' – a woman of Hispanic origin. Her success as a graduate student will ultimately help her become a role model in the community and bring badly needed diversity to the top level of biomedical profession.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change. *Nothing to Report.*

Actual or anticipated problems or delays and actions or plans to resolve them. *Nothing to Report.*

Changes that had a significant impact on expenditures. *Nothing to Report.*

Significant changes in use or care of vertebrate animals. *Nothing to Report.*

Significant changes in use of biohazards and/or select agents. *Nothing to Report.*

▪

6. PRODUCTS:

Journal publications.
1. Rollins DA, Coppo M and **Rogatsky I** Nuclear receptor coregulators of the p160 family: insights into inflammation and metabolism. (2015) *Mol Endocrinol* 29:502-17 *PMCID: PMC4399279* ((federal support acknowledged)
2. Sacta MA, Chinenov Y and **Rogatsky I** Glucocorticoid signaling: An update from a genomic perspective. (2016) *Annu. Rev. Physiol*. 78:155-80 (accepted in Sept 2015; federal support acknowledged)
-Two research papers are currently in review/revision.

Other publications, conference papers, and presentations.

10/2014 - Selected Speaker. Cold Spring Harbor Meeting. Nuclear Receptors & Disease, Cold Spring Harbor NY

(+ abstract from graduate student, Maria A. Sacta; selected for a poster presentation; and + abstract from Post-doctoral fellow Maddalena Coppo; selected for a poster presentation 10/2014; Cold Spring Harbor Meeting. Nuclear Receptors & Disease, Cold Spring Harbor NY)

03/2015 - Session Chair. The Endocrine Society 97th Annual Meeting, San Diego, CA
04/2015 - Seminar. Rutgers, The State University of New Jersey, Newark, NJ
05/2015 - Seminar. University College London, United Kingdom
08/2015 - Invited Speaker. FASEB Conference. Molecular and Systems Integration of Genomic and Nongenomic Steroid Hormone Action. Big Sky, MT

(+ abstract from graduate student, David A. Rollins; selected for an oral presentation and an award; 08/2015 FASEB Conference. Molecular and Systems Integration of Genomic and Nongenomic Steroid Hormone Action. Big Sky, MT)

09/2015 - Invited Speaker. EMBO Conference. Nuclear receptors: From Molecules to Humans. Ajaccio, France

(+ abstract from graduate student, Maria A. Sacta; selected for a poster presentation; 09/2015 - EMBO Conference. Nuclear receptors: From Molecules to Humans. Ajaccio, France)

Website(s) or other Internet site(s) *n/a*

Technologies or techniques
Any new protocols, databases and techniques that we develop in the course of this project will be publicly available following publication.

Inventions, patent applications, and/or licenses *n/a*

Other Products
Datasets from genome-wide analysis are in the process of being generated and will be publicly available when the project is completed and published.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Inez Rogatsky*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3.6*
Contribution to Project:	*Dr. Rogatsky is involved in planning the experiments, analyzing data, providing general oversight to the project progress.*
Funding Support:	*Additional Support is from the NIH and Rheumatology Research Foundation.*

Name:	*Maria A Sacta*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*9 (no compensation from this award)*
Contribution to Project:	*Ms. Sacta is the driving force of the project – she is responsible for all of the work described under 'Accomplishments' for tasks 2-3. She is also the heavy contributor to genome-wide analysis in Task 1*
Funding Support:	*NIH Diversity Supplement*

Name:	*Yurii Chinenov*
Project Role:	*Senior Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Chinenov handles the computational work involved in*

	genome-wide data analysis in Task 1.
Funding Support:	*Additional Support is from the NIH and Rheumatology Research Foundation.*

Name:	*Maddalena Coppo*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2 (no compensation from this award)*
Contribution to Project:	*Dr. Coppo performed RNA Pol II and GR ChIP-Seq experiments for this project.*
Funding Support:	*David Rosensweig HSS Genomics Center and the NIH*

Name:	*Rovena Pjetergjoka*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.5*
Contribution to Project:	*Ms. Pjetergjoka maintained the mouse colony and assisted with RT-qPCR*
Funding Support:	*Ms. Pjetergjoka was supported of the DOD award at 50% effort for approximately 10 months, the rest of the support was coming from the NIH and Rheumatology Research Foundation.*

Name:	*Bowranigan Tharmalingam*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.5*
Contribution to Project:	*Mr. Tharmalingam maintained the mouse colony and assisted with RT-qPCR*
Funding Support:	*Mr. Tharmalingam took over Research tech responsibilities in August 2015 and was supported by the DOD award at 50%, the rest of the support was coming from the NIH and Rheumatology Research Foundation.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Inez Rogatsky, PhD (PI): Changes in other support:
1) The Hospital For Special Surgery David Rosensweig Genomics Center (role: co-investigator; 5% effort; PI: Lionel B. Ivashkiv) – this support is no longer active
2) Barbara Volcker Center for Women and Rheumatic Disease (role: PI; 5% effort) - this support is no longer active
3) Rheumatology Research Foundation (role: PI, 25% effort) 07/01/14-06/30/16 - this support is now active.

Yurii Chinenov, PhD (Senior Research Associate): Changes in other support:

1) American Heart Association SGD #11SDG5160006 (role: PI, 44% effort) – this support is no longer active.
2) Barbara Volcker Center for Women and Rheumatic Disease (role: Senior Research Associate, 10% effort; PI: Rogatsky) - this support is no longer active
3) Rheumatology Research Foundation (role: Senior Research Associate, 20% effort; PI: Rogatsky) 07/01/14-06/30/16 - this support is now active.

What other organizations were involved as partners? n/a

8. SPECIAL REPORTING REQUIREMENTS: n/a

9. APPENDICES: (please see next page)

Glucocorticoid Signaling: An Update from a Genomic Perspective

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Keywords

glucocorticoid receptor, genomic approaches, transcriptional regulation, next-generation sequencing

Abstract

Glucocorticoid hormones (GC) regulate essential physiological functions including energy homeostasis, embryonic and postembryonic development, and the stress response. From the biomedical perspective, GC have garnered a tremendous amount of attention as highly potent anti-inflammatory and immunosuppressive medications indispensable in the clinic. GC signal through the GC receptor (GR), a ligand-dependent transcription factor whose structure, DNA binding, and the molecular partners that it employs to regulate transcription have been under intense investigation for decades. In particular, next-generation sequencing–based approaches have revolutionized the field by introducing a unified platform for a simultaneous genome-wide analysis of cellular activities at the level of RNA production, binding of transcription factors to DNA and RNA, and chromatin landscape and topology. Here we describe fundamental concepts of GC/GR function as established through traditional molecular and in vivo approaches and focus on the novel insights of GC biology that have emerged over the last 10 years from the rapidly expanding arsenal of system-wide genomic methodologies.

1. INTRODUCTION

Glucocorticoid hormones (GC) regulate three broad classes of normal physiological functions: (*a*) energy homeostasis, (*b*) embryonic development and postnatal life-history stage transitions, and (*c*) the stress response, thereby directly affecting survival and reproduction (1). GC production by the adrenal cortex cycles daily and seasonally and is under negative feedback control, whereby an elevated concentration of GC inhibits their production (2–4). Environmental and physiological stressors elevate GC levels, leading to a variety of adaptive physiological and behavioral changes to cope with stress-related increases in energy demands. As such, GC regulate the trade-off between energy allocation and energy-consuming functions, particularly when available resources are limiting. In that respect, increased GC production is anticipatory to meet the increased demand for energy expenditure during stress or life-history transitions. Similarly, GC reallocate metabolic resources to support immediate survival by inhibiting several energy-consuming processes such as digestion, reproduction, and inflammatory or immune response. With the cessation of stress, GC levels rapidly decline. Persistent or repeated stress results in sustained high GC levels and in the deregulation of negative feedback mechanisms that are mediated by the hypothalamic-pituitary-adrenal (HPA) axis. These changes are often maladaptive and may result in stress-induced pathologies (1, 5, 6).

Since the discovery of GC in the late 1940s, their potent immunomodulatory and anti-inflammatory activities have been actively exploited in the clinic and have largely shaped the investigation of GC actions. Indeed, studies of GC activities often focused on the two main medically relevant directions: suppression of inflammatory and immune responses and the side effects of the sustained exposure to pharmacological concentrations of GC. Notable side effects of prolonged GC therapies include metabolic changes that lead to type 2 diabetes and catabolic changes that result in atrophy or wasting of the skin and musculoskeletal system. Eventually, mechanistic information on GC signaling led to the structural characterization of GC receptors (GR), elucidation of their roles in gene regulation, and identification of both GR target genes and functionally important GR protein cofactors. Mechanistic studies, however, often employed saturating hormone concentrations and high-affinity ligands—these experimental setups fail to mimic physiological conditions, in which there is varying availability of the GC hormone and a repertoire of accessory proteins that further modulate response to GC (7). Thus, we are faced with a gap between the molecular and mechanistic findings obtained in reductionist systems and the historical observations in animal models and human patients.

The development of next-generation sequencing methods radically expanded the arsenal of analytical tools that capture the complexity of biological responses. In conjunction with standard biochemical and physiological methods, next-generation sequencing provides a quantitative inventory of specific biological molecules (e.g., mRNA, miRNA) or events (protein binding to DNA or RNA, nucleic acid modifications, DNA sequence variations) at the whole-genome level. Here, we begin by briefly reviewing the established physiological activities of GC and then focus on the emerging global roles of GC and their receptors uncovered through system-wide genomic approaches.

2. RECEPTORS FOR GLUCOCORTICOIDS

Biological activities of GC are mediated by two nuclear receptors (NR), the mineralocorticoid receptor (MR, aka NR3C2) and the glucocorticoid receptor (GR, aka NR3C1). In the absence of ligand, both receptors remain in the cytoplasm as part of multiprotein complexes containing chaperones and immunophilins (see Reference 8 for a review). Upon ligand binding, the receptors change conformation and translocate into the nucleus, where they either bind DNA sequences

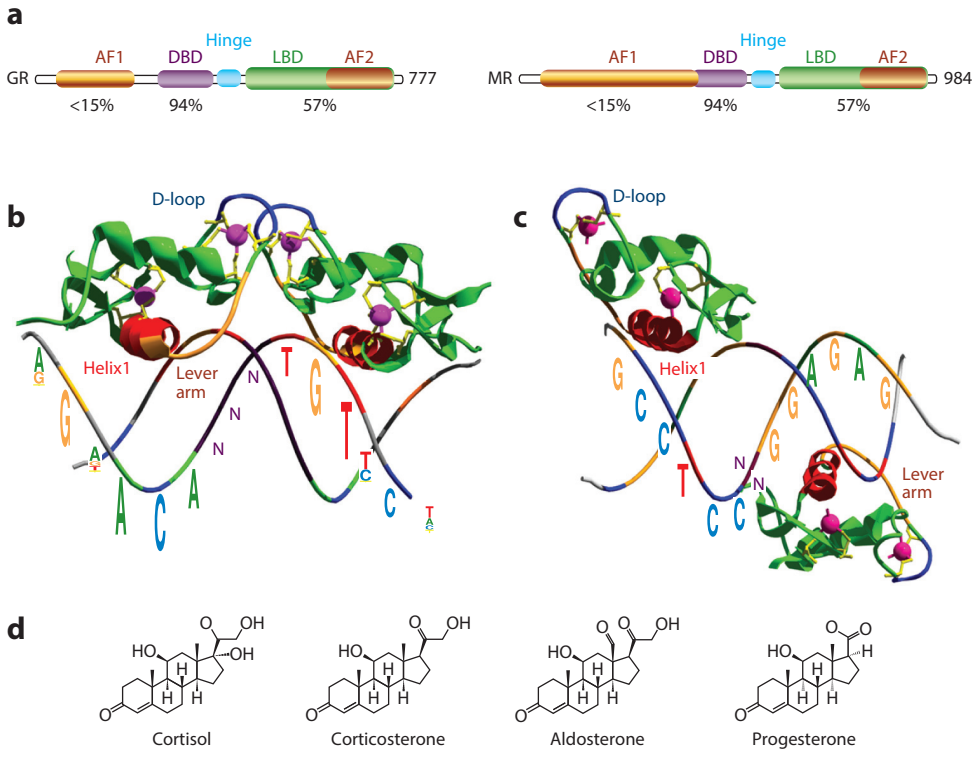

Figure 1

(*a*) Glucocorticoid and mineralocorticoid receptors (GR and MR) are two highly similar proteins that share structural organization, including the DNA binding and ligand binding domains (DBD and LBD) and two activation functions (AF1 and AF2). (*b*,*c*) The DBD of GR belongs to the C4 Zn-finger family that bind palindromic sites as dimers (*b*) (PDB:3G9P) or as monomers (PDB:4HN5) (*c*), depending on the distance between half-sites (see Section 4 for indicated structural elements). (*d*) GR and MR recognize several natural ligands.

known as GC and MC response elements (GRE and MRE, respectively) (**Figure 1**) or interact with other DNA-bound transcription factors to activate or repress transcription.

The production of and physiological response to GC are affected by a variety of factors, including (*a*) the systemic ligand concentration that changes rhythmically every 24 h, (*b*) local GC synthesis and processing that alter the ratio between biologically active and inactive ligands, and (*c*) the relative amounts of GR and additional DNA binding proteins and cofactors. Further complexity is introduced by a number of GR splice and translational isoforms, each with distinct biochemical properties (reviewed in Reference 4). Finally, GR and MR are subject to a variety of posttranslational modifications—including phosphorylation, sumoylation, ubiquitination, and acetylation (reviewed in References 9 and 10)—which alter their function.

GR and MR arose early in the evolution of vertebrates as a result of ancestral gene duplication circa 450 Mya (11, 12). These two receptors share highly similar DNA binding domains (DBDs) (94%) (**Figure 1*a***) and, at least in vitro, interact with similar palindromic sequences composed of two hexameric half-sites AGAACA separated by three base pairs (6 + 3 + 6) (**Figures 1*b*** and **3**) (13). Additionally, binding sites with variable linker lengths between half-sites were recently reported (14, 15). GR and MR DBDs belong to the C4 subfamily of Zn-finger DBDs (PFAM: PF00105),

and their crystal structures complexed with specific DNA have been solved (**Figure 1*b***) (16, 17). Besides interactions with DNA, the DBD mediates receptor dimerization and interactions with bZIP transcription factors, including those of the AP1 family (18, 19). The short hinge region connects DBD and the C-terminal region that encompasses a ligand binding domain (LBD) (**Figure 1*a***), which forms the activation function 2 (AF2) upon steroid binding. The GR and MR LBD regions also share a high degree of similarity (57%) and mediate interaction with a variety of chaperones that maintain the unliganded receptor in a conformation competent for ligand and coregulator binding. The N-terminal domain varies across the NR family, with only 15–20% similarity between GR and MR, and contains the activation function 1 (AF1), which mediates ligand-independent cofactor interactions (**Figure 1*a***) (9). In GR, phosphorylation of this intrinsically disordered domain induces an α-helical conformation that has been associated with transcription activation (20).

GR and MR recognize several ligands, including cortisol, corticosterone, aldosterone, and progesterone, but binding affinities and biological activities of different ligands vary (**Figure 1*c***): Cortisol, corticosterone, and aldosterone are agonists for both receptors, whereas progesterone is a competitive MR antagonist. MR has a tenfold-higher affinity for cortisol than for aldosterone and a tenfold-higher affinity for cortisol and corticosterone relative to GR. Paradoxically, early in vitro experiments reported that GR is a more potent transcriptional activator than MR (21, 22). Notably, GC levels in circulation even in unstressed organisms exceed that of aldosterone 10–100 fold, which led to the hypothesis that MR is a major transcription factor that is activated at basal levels of GC, whereas GR becomes engaged only in response to stress and at the zenith of circadian cycle, when the GC plasma level is highest (10). Interstitial concentrations of GC often do not correlate with total plasma levels due to tissue-specific mechanisms that either restrict bioavailability or stimulate local GC synthesis (23, 24), thus establishing local microenvironments dominated by one hormone or the other.

3. CENTRAL REGULATION OF GLUCOCORTICOID PRODUCTION

GC are produced by the adrenal cortex (in birds and mammals) or interrenal glands (in fish, amphibians, and reptiles); these tissues are responsible for maintaining the circulating level of GC. GC production changes rhythmically during the 24-h diurnal cycle that is superimposed upon a series of much shorter burst-like production spikes (7–13 per 24 h) referred to as the ultradian rhythm (25, 26). Circadian secretion of GC is imparted by the retinohypothalamic tract, which begins with light stimulation of retinal cells in the eye and ends in the suprachiasmatic nucleus (SCN) of the hypothalamus (**Figure 2*a***) (27, 28). SCN activation of the sympathetic nervous system/splanchnic nerve stimulates GC synthesis in the adrenal cortex to produce cortisol in humans and corticosterone in rodents (27, 29). Indeed, surgical lesions of the SCN impair diurnal regulation of GC secretion (27, 30). In addition, the SCN indirectly activates the HPA axis by producing corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These small peptide hormones stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) to then elevate production of GC in the adrenal cortex (**Figure 2*a***). High levels of plasma GC feed back to inhibit further production of CRH and ACTH by repressing transcription of *Crh* and *Pomc* (an ACTH precursor) (**Figure 2*a***) (4, 28). The negative feedback loop in the HPA axis forms a biological oscillator that regulates ultradian rhythmicity of hormone secretion within the 24-h cycle (2, 31) and leads to cyclic GR-mediated transcriptional regulation (26, 32–34).

At the molecular level, the shorter ultradian cycles of GR binding/exchange at the DNA are rapid and occur shortly after ligand washout (<10 min). Using a cell line with an integrated

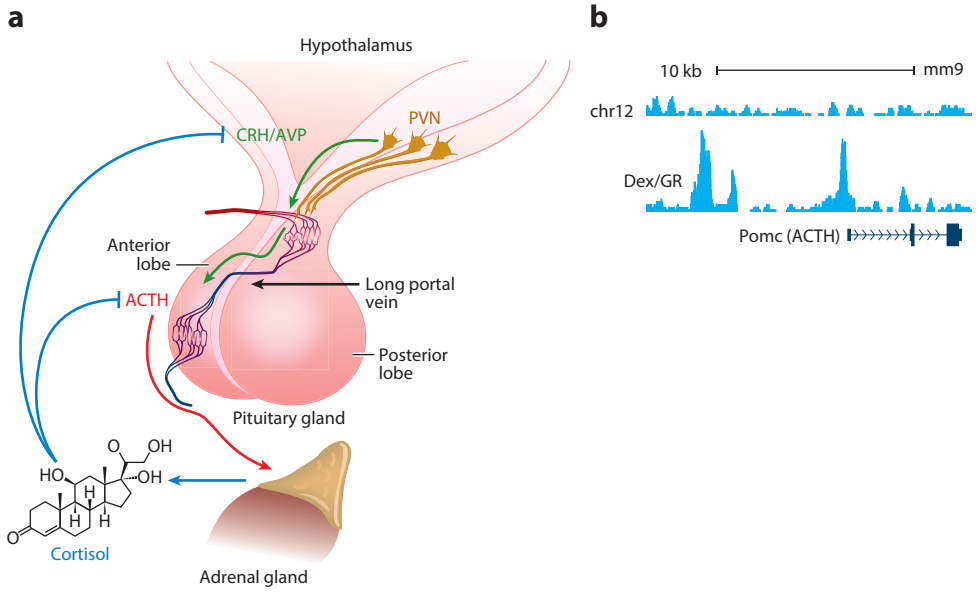

Figure 2

(*a*) Central regulation of glucocorticoid hormone production. (*b*) Two GR binding events at the *Pomc* gene, which encodes the ACTH precursor in the pituitary cell line (GEO:GSE37235). Abbreviations: ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; GR, glucocorticoid receptor; PVN, paraventricular nucleus of the hypothalamus.

array of multimerized GRE (an MMTV promoter array), Stavreva and colleagues (32) observed decreased mobility of DNA-bound GR at ultradian peaks and increased GR mobility upon hormone withdrawal. Similar GR dynamics were seen at the endogenous GR binding sites (GBS) in the *GluL* and *Mt1* genes, suggesting that GR loading on downstream target genes follows ultradian patterns (32). At the genome-wide level, transient hormone stimulation induced DNase-hypersensitive sites (DHS) that were rapidly lost upon hormone withdrawal. Conversely, many hormone-stimulated preexisting DHS persisted, suggesting a mechanistic basis for the molecular memory of past hormonal stimulation that may potentially prime transcriptional machinery for a faster response upon restimulation (35). Interestingly, both ultradian and circadian rhythms of GC are disrupted in diseases such as rheumatoid arthritis, depression, and sleep apnea (26, 36, 37). Additionally, patients with Cushing's syndrome—in which endogenous GC are overproduced—also have a shorter ultradian pattern of hormone secretion that may contribute to the metabolic and cardiovascular manifestations of Cushing's syndrome. Similar observations were reported for patients on long-term synthetic GC therapy (38, 39).

4. GLUCOCORTICOID RECEPTOR DIMERIZATION AND DNA BINDING

Genomic GBS (**Figure 3**) in vivo are broadly classified as simple, tethering, and composite. Simple sites are bound specifically by GR alone via a GR binding motif. Tethering sites recruit GR via protein-protein interactions with heterologous DNA-bound transcription factors. Finally, at composite sites GR binds cooperatively with additional DNA-bound factors. Genome-wide analyses of transcription factor binding identify short (200–1,000-bp) regions that are considerably

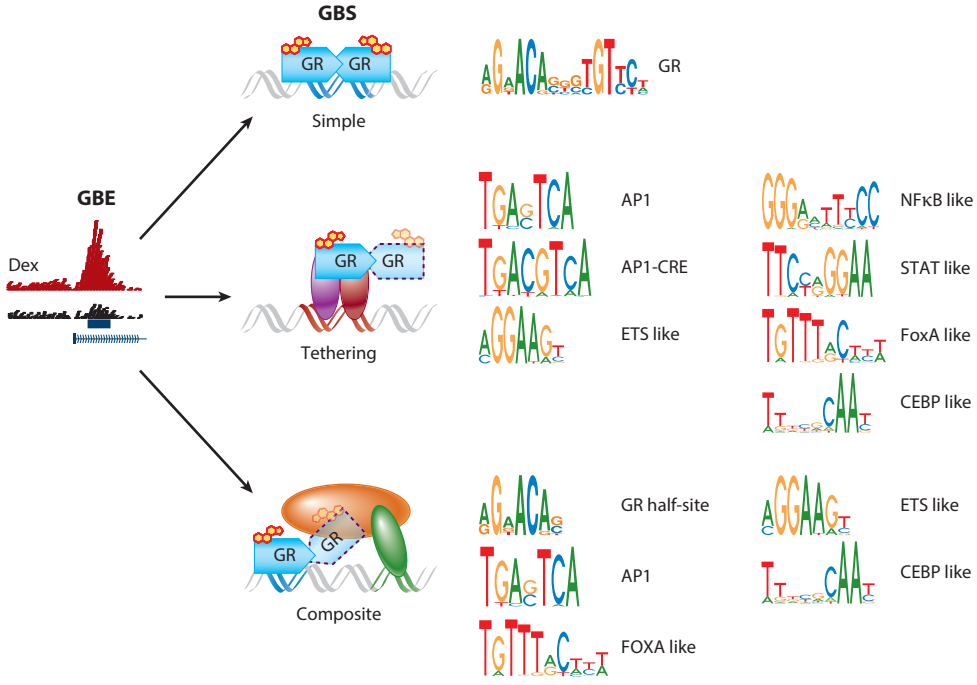

Figure 3

Types of genomic dexamethasone-dependent glucocorticoid receptor (GR) binding events (GBE) and DNA binding motifs overrepresented in the vicinity of GR binding sites (GBS).

wider than short binding motifs and that are typically centered at actual binding sites. In this review, we refer to these regions as GR binding events (GBE) (**Figure 3**), as it is impossible to identify the mode of GR binding within a given GBE without additional computational and experimental analyses.

The oligomeric status of GR in cells on and off DNA has been a subject of intense investigation. Early biochemical and structural experiments found that purified GR is bound as a homodimer to 15-bp palindromic DNA fragments and that residues within the GR Zn fingers confer interaction with DNA and homodimerization (**Figure 1*b***) (13). In the absence of DNA, full-length human GR bound the synthetic ligand triamcinolone acetonide (TA) predominantly as a monomer (40). Moreover, analyses of GR reporter activity and GR DNA residence time in vivo demonstrated an extremely rapid rate of GR turnover on DNA, which is difficult to reconcile with the existence of a preformed dimer (41–43). Finally, a recently reported high-resolution structure of GR bound to negative GBS with a shortened linker between hexameric half-sites suggested that GR not only can accommodate a wide variety of binding sites but also can bind DNA as a monomer, although with reduced affinity (15). This finding was corroborated by genome-wide analysis that revealed GR binding as a monomer or as a heterodimer with additional transcription factors bound to nearby sequences (44–46). Recent advances in quantitative high-resolution microscopy now enable one to track the behavior and oligomerization states of molecules in living cells. Using the number-and-brightness method, which relies on variations in intensity distributions between monomers and homo-oligomers (47), Presman and colleagues (48) reported that GR-eGFP exists as a dimer. However, whether this GR was truly DNA free was unclear. Another approach that relies on reflected light-sheet microscopy estimated that the slow-diffusing molecules that were

interpreted as DNA-bound GR constituted ~37% of nuclear GR (49). As GR dimerization is concentration driven, its oligomerization state likely varies depending on the availability of the receptor, coactivators, and unobstructed GBS in chromatin.

With respect to binding motifs, although GR binds a classic pseudopalindromic (6 + 3 + 6) GBS, many functional sites deviate from this rule. GR conformational flexibility is sufficient to adapt to a large number of palindromic sites (6 + [0–4] + 6) and, depending on the context, even to half-sites (6 + 0 + 0). Structural studies indicate that not only the oligomerization state but also the conformation of the GR DBD is altered upon DNA binding, often in a site-specific manner (15, 16, 50, 51). Indeed, it has been proposed that DNA acts as an allosteric ligand that alters GR conformation and, hence, receptor interactions with DNA and other proteins such as cofactors, ultimately affecting the transcriptional output. In fact, a comparison of several GR DBD crystal structures—in solution or bound to 13 different GBS—revealed site-specific conformational variations in DNA recognition α-helix H1, in a flexible loop between α-helix H1 and the second Zn finger (the lever arm), and in the D-loop (**Figure 1*b***) (50, 51). Importantly, both the half-sites and the linker contributed to establishing GBS-specific conformations of the DBD. Such conformations, together with changes in the dimerization interface, resulted in cooperativity between GR monomers and, ultimately, in differential regulation of transcription (51). Conversely, at unconventional palindromic GBS (6 + 1 + 6) that have been associated with transcriptional repression (14), GR assumed an alternative binding mode that precludes interactions between dimer interfaces of adjacent molecules bound to half-sites (**Figure 1*c***). At such sites, GR exhibited strong inter–half-site negative cooperativity, whereby binding of the first monomer to a high-affinity half-site attenuated the binding of another monomer (15). Thus, GBS-imposed oligomerization, orientation, and conformation of GR influence interaction surfaces for GR cofactors and specify appropriate, sometimes reciprocal, transcriptional outcomes.

In addition, the plasticity of the GR DBD allows the receptor to function at composite sites that contain binding motifs for GR and unrelated DNA-bound transcription factors (reviewed in References 52 and 53). The precise arrangements, affinities of binding sites, and interfactor interactions on composite elements are gene specific (52). It is generally assumed that binding by either partner to a composite site is weak and transient or insufficient to regulate transcription due to a failure to recruit or stabilize relevant cofactors, whereas cooperative interactions are expected to stabilize regulatory complexes. Such mechanisms are not unique to GR or other NR but reflect a common theme that has been previously described for several transcription factors, including ETS, bZIP, and Rel (54–57). We are only beginning to scratch the surface of our understanding of cooperative interactions between GR and other transcription factors. Introduction of whole-genome methods for binding site interrogation identified (*a*) DNA sequences enriched near GBS that are similar to binding sites of known transcription factors and (*b*) a variety of short sequences whose functional assignment remains to be done.

Finally, many genomic GBS lack discernable GR binding motifs and represent so-called tethering sites to which GR is recruited by other DNA-bound factors (**Figure 3**). Transcription factors from AP1, NFκB and STAT families, p53, SMAD3, and C/EBPα recruit liganded GR to their own binding sites. In the context of DNA-bound AP1 and NFκB, GR recruitment is typically associated with the repression of target genes (reviewed in References 53 and 58). Conversely, in the context of STAT5 binding sites, the GR:STAT5 complex activates transcription (59, 60).

The mechanisms of GR-mediated transcriptional regulation at tethering sites are likely diverse and gene specific. In the context of tethering site–bound complexes, GR may assume a conformation that promotes the recruitment of specific cofactors (61, 62). For example, GR-mediated repression of NFκB-driven genes requires GR cofactor GRIP1 (*Ncoa2*) and involves interference with either PolII recruitment to transcription start sites (TSS) or PolII pause release and early

elongation, depending on the gene (63, 64). It has been recently proposed that a subset of tethering sites contain adjacent GR half-sites (6 + 0 + 0) that initially promote weak GR binding (46). Indeed, an enrichment of GR half-sites in the vicinity of STAT3 binding sites was reported (65). The half-site facilitated tethering hypothesis (46) is consistent with the facilitated diffusion model (66) and with recent real-time in vivo analysis of chromatin association behaviors of transcription factors (67). Initial weak DNA binding by GR in a binding site–rich environment may facilitate further interaction with partnering proteins by acting as a molecular trap that nucleates formation of higher-order regulatory complexes. Whether GR is the first factor to bind or is recruited to such weak sites by prebound partnering factors is likely to be site specific. The half-site facilitated tethering hypothesis, although compelling, requires additional evidence demonstrating the importance of nearby half-sites in GR recruitment. If confirmed experimentally, this model will further corroborate the role of weak GR:DNA interactions and facilitated diffusion in stabilizing transcription factor complexes and will suggest a greater functional and perhaps structural similarity between GR complexes formed on tethering and composite sites.

5. GENOME-WIDE ANALYSIS OF GLUCOCORTICOID RECEPTOR BINDING

The development of next-generation sequencing and, in particular, ChIP-seq and related methods (FAIRE, DNase-seq, ChIP-exo) has led to a paradigm shift in our understanding of the regulation of gene expression. System-wide analyses of protein–nucleic acid interactions coupled with genome-wide assessment of gene expression introduced a comprehensive and integrated approach to studying biological phenomena. The high sensitivity of these methods can now be leveraged to define the molecular signatures of GR actions unique to specialized cell types or tissues [see, for example, a GR ChIP-seq study in hippocampal neurons (68)].

As of May 2015, more than 20 GR cistromes in humans, mice, and rats have been published, allowing for an extensive comparative analysis of GR DNA binding and GR-mediated activities in a variety of cell types and organs (see **Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). The number of reported hormone-induced GBE or DHS varies from a few thousand to as many as ~70,000 per genome, with the median at ~9,000 in humans and mice. This number is affected by multiple variables, including the statistical stringency of the peak-calling procedure, the duration of hormone stimulation, the relative levels of GR expression, and the type of ligand. Nonetheless, the number of hormone-induced events considerably exceeds the number of affected genes as determined by concurrent RNA-seq and microarray analyses. This scenario implies either that more than one binding event contributes to the regulation of a given gene or that the majority of binding events do not directly contribute to gene expression. As reported for other NR (73), a relatively small percentage (3–7%) of GBE occur near TSS, and most are evenly partitioned between intragenic (predominantly intronic) and distal intergenic regions. Intragenic GBE are typically enriched in the first intron (69, 70), which is consistent with the higher conservation and the larger regulatory potential of first introns (71). Finally, GBE tend to be clustered as closely spaced peaks (72).

The majority of hormone-induced GBE (69–82%) are detected within preexisting accessible chromatin, with far less (5–17%) detected in de novo remodeled chromatin (72, 74). Interestingly, in human cell lines, nearly all GBE that were detected using low ligand concentrations ($<K_d$) are located in preexisting open chromatin (75). Kinetic analysis of GR binding indicates that transient hormone stimulation, which mimics ultradian hormone pulses, triggers transient loading of GR that is rapidly dissociated upon hormone withdrawal (32, 33, 35). Prolonged hormonal exposure considerably expanded the number of engaged GBS, increased the accessibility of preexisting

Supplemental Material

DHS, and created novel DHS that were not apparent upon transient exposure. Interestingly, most dynamic DHS were associated with promoter-distant GBE that are likely to be involved in long-range interactions with TSS (35).

The reliance on open chromatin for GR binding has several important functional consequences. First, because the DHS repertoire is cell type specific, factors other than GR are needed to create and maintain open chromatin, implying an important role of partnering factors and lineage-determining enhancers in GR-mediated gene regulation. Second, due to the difference in chromatin accessibility between cell types, the repertoire of accessible GBS also differs. Indeed, a comparison of GBS in six different mouse and human cell lines revealed only a moderate 5–40% overlap (70, 74). Furthermore, of all (11,666) GBE reported by Grontved et al. (74) in the mouse liver, only 0.5% (~580) were found in all six cistromes, whereas more than 83% (9,500) were liver specific. Although some lack of consistency between these cistromes can be attributed to distinct peak-calling protocols, it appears more likely that prolonged hormone treatment leads to broad transcriptional reprogramming, especially in cell types in which GC cause differentiation or selection.

The interactions between GR and individual partnering transcription factors have been well characterized biochemically (52). However, genome-wide approaches provide a unique opportunity to identify such interactions on a global scale. Computational analyses of overrepresented sequence motifs near the GBS produce a list of candidate factors that could be experimentally tested. Alternatively, GBS positions can be correlated with genome-wide factor-specific binding events done in the same experiment or previously published. **Figure 3** shows several DNA motifs typically overrepresented near GBS. As expected, several versions of GR binding motifs—either palindromic sites (6 + 3 + 6) or half-sites (6 + 0 + 0)—are typically enriched near the peak summits of GBE. In addition, binding sites for AP1/CREB/ATF3, RUNX, SP1/KLF/ZNF, ETS/TEAD, C/EBP, FOXA (forkhead A), NFκB, and IRF3 are common.

In cooccupancy experiments, half of the hormone-induced GBE in the 3134 murine mammary epithelial cell line correlated with AP1 (JUN) occupancy (76). AP1 binding was hormone independent and was often associated with open chromatin. Furthermore, overexpression of a DNA binding–deficient mutant of FOS, a JUN dimerization partner, led to global changes in expression of GR-regulated genes, reduced chromatin accessibility, and reduced GR recruitment to such sites. This observation suggests that, at a subset of GBS, prior AP1 binding is required to maintain chromatin structure permissive for GR binding after ligand stimulation (76). Similarly, GR and NFκB (p65) cooccupy more than 1,000 sites in HeLaB2 cells after cotreatment of cells with TA, a synthetic GC, and TNF, an NFκB inducer. Most of these GBE contained either NFκB or AP1 binding motifs rather than classic GBS. GR recruitment to these sites was abolished after knockdown of p65, a component of the NFκB heterodimer, implying that p65 tethers GR to DNA. In functional studies, GBE cooccupied by both p65 and GR were linked to genes upregulated by TNF and repressed by TA (70). Similarly, a correlation between AP1, p65, and GR occupancy was reported in macrophages upon prolonged treatment with GC and stimulation with LPS (77).

Enrichment of AP1 and NFκB sites at GBE is common but not universal. For example, in mouse livers and in 3T3-L1 adipocytes, enrichment of the C/EBP binding motif is much higher than that of the AP1 motif. C/EBPβ ChIP-seq revealed that 62% of GBE in mouse livers and 67% of GBE in 3T3-L1 adipocytes overlapped with C/EBPβ binding sites (74, 78) and that GR and C/EBPβ facilitated each other's recruitment to DNA.

Recent development of the high-resolution ChIP-exo approach (79) has provided new insights into interactions between GR and partnering proteins. ChIP-exo combines ChIP with subsequent exonuclease digestion that trims fragments of immunoprecipitated DNA down to shorter fragments (~30 bp) protected by a cross-linked protein (45). The advantages of ChIP-exo are

near-single-base-pair resolution and the uniqueness of read distribution profiles (footprints) for a bound transcription factor. Composite sites generate a combined footprint with identifiable features contributed by individual proteins. Because the presence of cell-specific partnering proteins may alter GR footprint profiles, ChIP-exo is particularly suitable for detecting noncanonical binding sites created by cell type–specific partners. Indeed, a comparison of binding motifs enriched in GR ChIP-seq from three different cell lines, K562, IMR90, and U2OS, revealed that far fewer peaks in K562 cells contained a canonical 6 + 3 + 6 GR binding motif, suggesting that GR may be recruited to other sequences.

Comparison of footprint profiles in IMR90 cells identified several composite sites in which GR, likely as a monomer, bound together with partner proteins that belong to the ETS, FOX, and STAT families. Hormone-dependent loading of two ETS proteins, TEAD3 and TEAD4, in conjunction with GR has been confirmed for several composite sites, particularly at the *Nfia*, *Ptpn1*, and *Acpl2* genes (45). FOX binding motifs enriched in GR ChIP-seq peaks exhibited a footprint profile similar to FOXA1 profiles previously obtained by ChIP-exo (80). In the latter study, monomeric GR associated with binding motifs for the liver-specific transcription factors HNF4A, C/EBPβ, and ONECUT1 (HNF6), indicating that lineage-specific transcription factors may be preferentially involved in GR monomer stabilization at composite sites (46).

The analyses of GR cistromes has led to important new insights into novel GR binding properties and to an appreciation of the diversity and cell specificity of GBS. Although a canonical GR dimer at a palindromic GR binding motif remains an important entity, these newer studies have highlighted the functional significance of monomeric GR and GR cooperatively bound to composite sites with cell type–specific partnering factors (44–46). Indeed, composite sites that are bound by transcription factor complexes expand the repertoire of available interaction surfaces for additional cofactors, thus increasing the plasticity of responses to a variety of stimuli in a tissue- and factor-specific manner.

GR interacts with a vast number of coregulators through hormone-independent (AF1) and hormone-dependent (AF2) activation functions, with several cofactors binding the hinge region between the DBD and LBD (for reviews, see References 81–83). Cofactors regulate transcription through several mechanisms, including bridging of receptor-containing transcription complexes with general transcription machinery, posttranslational modification of histones and chromatin-associated proteins, and ATP-dependent chromatin remodeling (57, 84). Regulation of a single gene typically relies on multiple cofactors that are recruited or dismissed in an ordered manner. Cofactors are also targeted by a variety of enzymes (kinases, ubiquitin ligases, and acetyl- and methyltransferases), thus acting as integrators of a wide range of signals. GR cofactors include adaptor proteins from the p160 family (NCOA1/SRC1, NCOA2/GRIP1/TIF2/SRC2, and NCOA3/SRC3), histone acetyltransferases CBP and p300, histone methyltransferases (e.g., G9a), histone demethylases, and a multiprotein mediator complex. Of those, the biochemistry and functions of p160 coregulators in relation to GR and other NR have been described in the greatest detail. The three family members are structurally similar and contain conserved protein-protein interaction domains that recruit an extensive array of secondary cofactors. p160 coregulators associate with the GR AF2 via a centrally located NR-interacting domain that contains three LxxLL boxes. The biological roles of p160s, as revealed by mouse genetics, span many aspects of endocrine, metabolic, and circadian regulation and have been extensively reviewed (82, 83).

6. GLUCOCORTICOIDS AND REGULATION OF METABOLISM

The role of GC in metabolic regulation is perhaps best highlighted in patients with Cushing's syndrome. Prolonged exposure to cortisol in Cushing's syndrome can be caused by pituitary

adenomas, neuroendocrine tumors, or adrenocortical adenomas. Cushing's syndrome can also develop as a result of excessive administration of GC medications (85). In these patients, loss of the normal circadian rhythm of cortisol secretion leads to a plethora of metabolic abnormalities, including insulin resistance, dyslipidemia, and centripetal obesity (86). Excess GC promote gluconeogenesis in the liver and antagonize the effects of insulin on peripheral glucose utilization, thereby leading to glucose intolerance. Similarly, prolonged treatment with GC in laboratory animals and human patients is associated with fasting and postprandial hyperglycemia and reduced insulin sensitivity, with decreased insulin-mediated suppression of lipolysis, and with elevated proteolysis during hyperinsulinemia (87–89). Given the association between excess cortisol and metabolic disease, we review older and current genome-wide studies of GC/GR carried out in key metabolic organs.

6.1. Liver

Patients with GC excess present with hyperglycemia and insulin resistance—two major risk factors for the development of type 2 diabetes (90). The liver plays a central role in glucose metabolism and is a major target site for GC action. In the absorptive period (after a meal), the liver utilizes glucose for glycogen synthesis and for the generation of metabolic intermediates to be used in glycolysis and the TCA cycle. Insulin suppresses glucose production by inhibiting glycogenolysis and gluconeogenesis. In the postabsorptive period (during fasting), insulin drops, and net glucose production by the liver increases via gluconeogenesis (91).

In the liver, GC is essential for maintaining normal blood glucose levels during the postabsorptive period, when awakening, and during periods of stress, partly by increasing the hepatic responsiveness to glucagon (92). In newborn whole-body GR knockout mice, transcripts for two rate-limiting enzymes in gluconeogenesis, *G6pc* and *Pck1*, which encode glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK), respectively, were reduced despite high circulating GC (93, 94). Furthermore, a conditional liver-specific deletion of GR (GRcKO) led to hypoglycemic 50% lethality within 48 h of birth (95, 96). In adult GRcKO mice, although glycogen content and levels of blood glucose, alanine, free fatty acids, and ketone bodies were similar to those of wild-type mice, insulin levels decreased 1.5 fold, and glucagon levels increased 2.1 fold, suggesting a compensatory mechanism. When these GRcKO mice were fasted for prolonged periods—during which gluconeogenesis becomes the major source of serum glucose—they displayed a faster decline in blood glucose, increased levels of circulating GC, and impaired induction of liver *Pck1* relative to wild-type mice. In the streptozotocin-induced model of type 1 diabetes, hyperglycemia and an increase in *Pck1* expression were consistently reduced in GRcKO compared with wild-type mice, suggesting that loss of GR is protective (95). Consistent with these findings, pharmacological inhibition of GR in *ob/ob* and *db/db* mice improved glucose tolerance and reduced PEPCK and G6Pase activity, thereby increasing insulin sensitivity in these two diabetic, obese mouse models (97, 98).

PEPCK, a rate-limiting enzyme in gluconeogenesis and a direct GR target, has been extensively studied. In addition to a GBS at −395 and −349 bp from the TSS, four other elements (AF1, AF2, AF3, and CRE), which bind COUP-TF/HNF4, HNF3β, COUP-TF, and C/EBPβ, respectively, are necessary for full *Pck1* gene activation (reviewed in Reference 99). ChIP in rat hepatoma cells revealed that all of these factors are recruited to the *Pck1* promoter region in response to GC and, importantly, dissociate upon insulin treatment, providing the molecular basis for the opposing physiological effects of GC and insulin.

Interestingly, later studies pointed to the role of other NR in *Pck1* regulation. Specifically, PPARα, whose expression is induced by dexamethasone in cultured hepatocytes and intact rodent

livers, appears to play a major role in steroid-induced diabetes. *Ppara* knockout mice failed to develop hyperglycemia or hyperinsulinemia after chronic dexamethasone treatment and had decreased *Pck1* and *G6pc* expression relative to wild-type mice (100). Genome-wide experiments recently confirmed the recruitment of PPARα to both genes (101, 102). Additionally, liver X receptor (LXR)β also appeared to be required for GC-induced hyperglycemia, as LXRβ knockout mice failed to develop both hyperglycemia and dexamethasone-induced insulin resistance and had reduced expression of *Pck1*, possibly due to failure to recruit GR to the *Pck1* promoter (103). The molecular mechanism of the cross talk between GR and LXR has not been investigated.

Furthermore, farnesoid X receptor (FXR), a bile acid sensor in enterohepatic tissues, has been linked to GR in regulating glucose metabolism in the unfed state. In particular, unfed FXR knockout mice were hypoglycemic and had decreased *Nr3c1* (GR), *Pck1*, and *G6pc* expression. FXR binding at −34.5 kb from the *Nr3c1* TSS was sufficient to increase GR expression, which in turn was required for the induction of *G6pc* and *Pck1* following FXR activation. This finding suggests that, in the unfed state, FXR sets into place the gluconeogenic program by regulating GR expression (104). Combined, these studies demonstrate a complex interplay between GR and NR activated by fatty acids (PPARα), bile acids (FXR), and oxysterols (LXRs), suggesting that the prevailing combination of NR affecting glucose homeostasis in the liver is likely determined by a specific metabolic state.

Genome-wide studies of GR occupancy in whole livers of adrenalectomized mice treated with dexamethasone also revealed an extensive cooccurrence of GBS, C/EBPβ binding sites, and DHS (74). Given that C/EBPβ regulates glucose and glycogen metabolism in the liver (105), its preoccupancy at GBS containing both C/EBPβ and GR half-sites is consistent with the idea that cooperative interactions between these factors are required for GR loading and ultimately for regulation of transcription (74). Indeed, disruption of C/EBPβ binding by overexpressing a dominant negative C/EBPβ mutant resulted in decreased chromatin accessibility in many preexisting DHS, in decreased GR recruitment, and in attenuated expression of several genes that otherwise corecruit C/EBPβ and GR (74).

Apart from gluconeogenesis, GC have also been implicated in the regulation of fatty acid metabolism in the liver. Patients with Cushing's syndrome often present with dyslipidemia, with an increase in triglyceride (TG) and total cholesterol levels (106). In a small study of Cushing's syndrome patients, a liver CT scan revealed that 20% (10/50) of them had nonalcoholic fatty liver disease (NAFLD) and increased visceral fat deposition (107). Conversely, a liver-specific disruption of GR in *db/db* mice by tail vein injection of adenovirus expressing GR-specific shRNA led to a pronounced reduction in hepatic TG and to elevated circulating ketone levels, consistent with an increase in fatty acid oxidation as the predominant source of energy (108). Also observed were downregulation of hepatic genes mediating lipid storage and transport [e.g., genes encoding caveolin 1 (*Cav1*), the fatty acid transporter *Cd36*, and microsomal TG transfer protein (*Mttp*)] and upregulation of genes involved in fatty acid oxidation and TG hydrolysis [e.g., genes encoding pancreatic lipase (*Pnlip*) and pancreatic lipase–related protein (*Pnliprp2*, *Plrp2*)]. Thus, loss of liver GR in these *db/db* mice ameliorated hepatic steatosis by increasing hydrolysis of TG stores. The same study proposed an intriguing connection between GC-dependent regulation of TG metabolism in the liver and HES1, which is a transcriptional repressor activated downstream of Notch signaling (108). GR represses *Hes1* by directly binding to negative regulatory elements in the *Hes1* promoter at −463 and −414 relative to TSS concomitant with the recruitment of histone deacetylases (108). An alternative mechanism proposed for GC-mediated repression of *Hes1* involves GR tethering to NFκB at the first intron of the *Hes1* gene (109). In this study, the regulation was bidirectional, as the HES1 protein in turn repressed a large subset of GR targets

in several human lines. Indeed, the liver-specific disruption of *Hes1* dramatically potentiated GC-mediated induction of *Igfbp1*, *Pck1*, and *G6pc* and was associated with glucose intolerance (109).

Despite ample biochemical and molecular evidence for the role of GR and its partners in the regulation of metabolic processes in the liver, the exact mechanisms and targets that fine-tune glucose and TG metabolism in response to GC in vivo are not well understood. A caveat of existing studies is the reliance on loss-of-function approaches and potential indirect effects of GR depletion on the HPA axis and linked metabolic processes. Furthermore, most of these studies utilize synthetic GR ligands with much higher affinity for GR and, conversely, lower affinity for corticosteroid-binding globulin in plasma. Consequently, these compounds achieve a much higher concentration and bioavailability in tissues, leading to an artificially enhanced or prolonged effect on GC-responsive genes.

6.2. White Adipose Tissue

During the absorptive period, excess energy is stored in the form of TG in white adipose tissue (WAT). During fasting or exercise, GC, growth hormone, and catecholamines cause the breakdown of TG, and fatty acids are released into circulation to be used by the liver for gluconeogenesis (110). Physiologically, GC promote preadipocyte differentiation into mature adipocytes and increase caloric and dietary fat intake. GC also drive the hydrolysis of TG by increasing the expression and activity of hormone-sensitive lipase (111, 112), and increase de novo lipid production in hepatocytes (108). GC have been described as either anti- or prolipolytic in various mouse studies (110, 113). Furthermore, Cushing's syndrome patients present with marked centripetal obesity, suggesting that GC regulates both adipocyte differentiation and lipolysis. Indeed, two human studies report that centripetal obesity in Cushing's syndrome or induction of hypercortisolism results from increased abdominal fat depots with a concomitant decrease in lipolytic activity (112, 114).

In mouse 3T3-L1 preadipocytes cortisol increased lipolysis in a dose-dependent manner (115). Moreover, genome-wide GR drove a lipolytic transcriptional program during adipocyte differentiation, activating multiple genes involved in lipid metabolism—e.g., *Dusp1* (dual-specificity phosphatase 1, also known as MKP1), *Lcn2*, *Pik3r1*, *Sgk1*, and *Tsc22d3*—which correlated with GR loading on associated GBS (115). Furthermore, as an in vivo correlate, the authors found that a panel of 14 genes involved in TG homeostasis were induced in the inguinal fat of mice following a 4-day treatment with dexamethasone (115). These genes include those involved in TG synthesis (*Scd1–3*, *Agpat9*, *Agpat2*, and *Lipin1*), lipolysis (*Lipe* and *Mgll*), lipid transport (*Cd36*, *Lrp1*, *Vldlr*, and *Slc27a2*), and lipid storage (*Plin4*). Except for *Agpat2*, *Scd3*, and *Slc27a2*, all of these genes had at least one GBS that conferred hormonal response. Consistently, in vivo, both TG synthesis and lipolysis were enhanced in dexamethasone-treated mice (115). Of note, more studies are necessary to discriminate between the GC-dependent TG synthesis and breakdown because, depending on the concentration and duration of treatment, both effects can be achieved (113).

With respect to the role of GC in adipocyte differentiation, studies in both mouse and human systems point to a requirement for GC signaling. In mouse 3T3-L1 preadipocytes, GC are an essential component of the differentiation cocktail, which also contains insulin, the phosphodiesterase inhibitor methylisobutylxanthine (IBMX), and fetal bovine serum. Dexamethasone primes preadipocytes and sets the stage for sequential induction of various regulators of adipogenesis, including C/EBPδ, C/EBPβ, C/EBPα, and PPARγ. Consistently, differentiation of human mesenchymal stem cells into adipocytes was attenuated if the GR antagonist RU486 was added to the cocktail mix. This inhibition was attributed to blunted induction of *Klf15*, a GR-activated gene that contributes to the expression of C/EBPα and PPARγ (116).

An independent study that used fibroblast-like human preadipocytes (SGBS cells) as a model for adipocyte differentiation revealed a panel of NR genes dramatically affected by GC. These included upregulated *AR*, *PPARG*, and *NR1H3* (Lxra) and downregulated *RARG*, *PPARD*, *NR1D1* (Rev-erba), *NR1D2* (Rev-erbb), *VDR*, and *NR3C1* (GR) within the first 4–8 h of differentiation. In fact, many of these genes, including *PPARG*, *NR1D2*, *VDR*, and *NR3C1*, have GBS near the TSS (117). Notably, similar gene expression changes were seen in the course of mouse preadipocyte differentiation (118). Of particular importance, IBMX and GC were necessary for the upregulation of *PPARG*, an established master regulator of adipocyte differentiation (118, 119).

Genome-wide DNase-seq and ChIP-seq studies also showed widespread GR binding to the genome in 3T3-L1 preadipocytes within 4 h of differentiation (119). Most DHS corresponding to GBE were transient; however, some persisted throughout differentiation, positioning GR as a priming factor for later binding of other transcription factors at these sites. Importantly, C/EBPβ binding was also most prominent at 4 h and partially overlapped with GBE at this time. Similar to findings in the liver, C/EBPβ in 3T3-L1 preadipocytes functionally interacted with other transcription factors such as GR, STAT5, and retinoid X receptor (RXR) to elicit differentiation (119). Interestingly, a subset of DHS that overlapped with C/EBPβ binding sites retained their open chromatin structure throughout differentiation and then bound PPARγ. Importantly, GR, C/EBPβ, STAT5, and RXR binding to these remodeled hot spots during early adipogenesis correlated highly with PolII occupancy at nearby genes and with transcriptional activation; both STAT5 binding and transcriptional activation were prevented by GR knockout (119). In an independent study, GR and C/EBPβ colocalized at enhancer sites with high H3K9 acetylation occupied by p300 and MED1 and facilitated each other's recruitment (78). Importantly, expression of *Pparg* in the early stages of adipogenesis depended on binding of both GR and C/EBPβ to enhancer sites of *Pparg* (78).

Given the effects of GC on lipogenesis and lipolysis as well as on adipocyte differentiation, it is not surprising that excess GC deregulate the balance between TG storage and breakdown. Conditions of excess GC such as Cushing's syndrome and metabolic syndrome are predicted to drive the differentiation of visceral adipose tissue, leading to centripetal adiposity. Furthermore, a recent study showed that, in a proinflammatory environment when adipocytes are exposed to TNF, GR is activated in a ligand-independent manner, leading to the upregulation of VDR, which, together with GR, drives a transcriptional program of insulin resistance (120).

6.3. Muscle

In skeletal muscle, GC regulate protein and glucose metabolism. During the absorptive period, insulin stimulates the uptake of glucose by skeletal muscle, where glucose is stored as glycogen. In the postabsorptive period, catecholamines, glucagon, and GC promote glycogenolysis. Increased GC levels in Cushing's syndrome or GC-treated patients lead to insulin resistance and inhibit protein synthesis while promoting proteolysis to provide amino acids for gluconeogenesis; these events ultimately lead to skeletal muscle atrophy and weakness (121, 122). Indeed, pathological conditions associated with high circulating levels of GC promote catabolic pathways in skeletal muscle and muscle atrophy (122–124).

The balance between anabolic and catabolic pathways in skeletal muscles is regulated by several interdependent pathways that ultimately converge on the protein kinase AKT (125). When activated, AKT increases protein synthesis via the mTOR serine/threonine kinase pathway (the anabolic arm) and decreases protein degradation by phosphorylating and retaining the forkhead transcription factors FOXO1 and FOXO3 in the cytoplasm (the catabolic arm) (**Figure 4**). These FOXO transcription factors upregulate several genes encoding enzymes involved in proteolytic

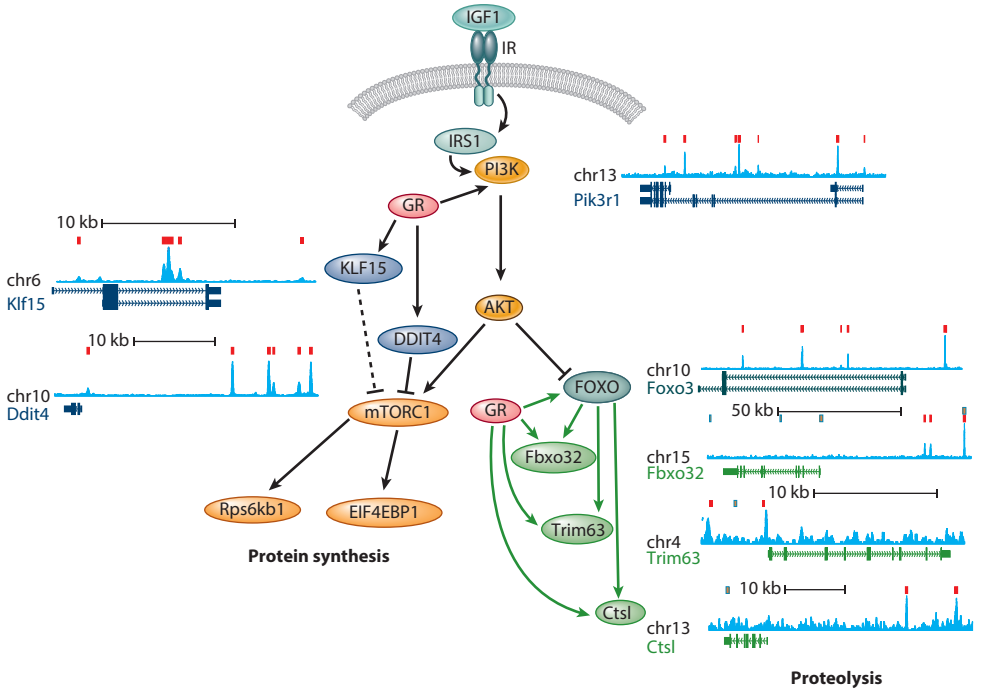

Figure 4

GC-mediated regulation of protein synthesis and degradation in the muscle. GR activates the transcription of several genes whose products inhibit the AKT-mTORC1 pathway (*Ddit4*, *Klf15*) and facilitate protein degradation (*Fbxo32*, *Trim63*, *Ctsl*). In conjunction with transcription factors from the FOXO family, GR forms putative coherent feed-forward loops regulating a set of common target genes (*green arrows*). GBE (*blue profiles* and *red bars*; GEO:GSE46116) and FOXO binding events (*orange bars with blue borders*; GEO:GSM1175114) were extracted from GEO data sets and were visualized in the UCSC genomic browser (**http://genome.ucsc.edu**). Abbreviations: GC, glucocorticoid hormone; GR, GC receptor; IGF1, insulin-like growth factor 1; IR, insulin receptor; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase.

pathways, including two E3 ubiquitin ligases, Fbxo32 (*atrogin-1*, *MAFbx*) and Trim63 (*MuRF-1*) (reviewed in Reference 126), and a lysosomal cysteine protease (*Ctsl*), implicated in myofibril necrosis (127). AKT activity is regulated by several upstream signals, including insulin and insulin-like growth factor 1 (IGF1), which are typically growth promoting. Specifically, insulin binding by the insulin receptor (IR) induces IR tyrosine autophosphorylation and phosphorylation of the insulin receptor substrate 1 (IRS1). IRS1 is an adaptor protein that recruits and activates phosphoinositide 3-kinase (PI3K), which eventually activates AKT (126, 128). Conversely, GC target several control points in the AKT pathway that ultimately decrease AKT activity and concurrently decrease protein synthesis and increase protein degradation (**Figure 4**).

Genome-wide analysis of GR binding in dexamethasone-treated C2C12 myocytes revealed 2,251 GBE; most of these GBE were located in introns (42%) and intergenic regions (29%), with only 5% located within 5 kb upstream of the TSS (129). Expression profiling revealed that of 363 upregulated and 218 downregulated genes only 147 and 26, respectively, had associated GBE. Gene ontology analysis identified genes involved in the receptor tyrosine kinase pathway (e.g., *Cblb* and *Pid1*, which inhibit IRS1 activity), genes encoding inhibitors of mTOR signaling (e.g., *Sesn1* and *Ddit4*), and genes implicated in the pathogenesis of insulin resistance (e.g., *Pik3r1*,

which encodes the regulatory p85α subunit of PI3K) (129). GR bound to several GBS near *Pik3r1* (**Figure 4**) (129), and interestingly, increased p85α expression in myotubes inhibited PI3K activity, which correlated with smaller myotubes and an upregulation of FOXO targets associated with muscle atrophy (129). GR also directly activates atrophy-associated catabolic genes by binding to several intronic and upstream GBS in *Trim63*, *Fbxo32*, *FoxO3*, and *Klf15* (130–133).

Finally, GR induces *Ddit4* (REDD1), a repressor of the mTORC1-mediated anabolic pathway in muscle (**Figure 4**) (134). The mTORC1 kinase is a signal integrator for multiple regulatory cues—including growth factors; energy status; amino acids; and, in particular, branched-chain amino acids (BCAA). As with catabolic activators, GR directly binds a cluster of far-upstream *Ddit4* enhancers that mediate robust and rapid induction of *Ddit4*. DDIT4 inhibits RHEB, a GTPase and positive regulator of mTORC1, thus blunting mTORC1 activation and leading to a decrease in protein synthesis (134). Consistent with the GR regulation of *Ddit4*, whole-body REDD1-null mice are protected from dexamethasone-induced muscle wasting (135). Curiously, GC-induced KLF15 also activates transcription of branched-chain aminotransferase 2, a mitochondrial enzyme that catalyzes the first reaction in the catabolism of BCAA. Thus, dexamethasone-mediated upregulation of KLF15 affects the balance of anabolic and catabolic pathways both directly, by promoting protein degradation, and indirectly, by decreasing the availability of the natural mTOR activator (132).

7. GLUCOCORTICOIDS AND CONTROL OF INFLAMMATION

In the clinic, GC have been used as potent immunosuppressive and anti-inflammatory drugs for more than 60 years. Inflammation is a protective response to infection, irritation, or injury aimed at abating insulting stimuli, clearing pathogens, initiating tissue repair, or engaging the adaptive immune system when needed. These processes rely on several types of innate immune cells, including macrophages, dendritic cells, neutrophils, and mast cells. Such innate immune cells perform specific functions in threat recognition, initiation and propagation of the inflammatory reaction, communication with cells of the adaptive immune system, and eventually resolution of inflammation. Inflammation is typically initiated when specialized pattern recognition receptors such as Toll-like receptors, Rig-I–like receptors, Nod-like receptors, C-type lectin receptors, and cGAS sensors (reviewed in Reference 136) of the host innate immune cells recognize danger signals (137). Once engaged, these receptors activate numerous signal transduction pathways that eventually converge upon a few transcription factors from the AP1, NFκB, STAT, and IRF families that together activate a broad transcription program aimed at amplifying and sustaining the inflammatory process.

A correlation between endogenous GC levels and inflammation has been noted for more than 40 years (138). However, mechanistic insights into the anti-inflammatory activities of GC were firmly established by the development of GRKO mice. When GR was conditionally deleted in macrophages, GRmKO mice displayed higher sensitivity to and poorer survival following LPS-induced endotoxin shock and elevated serum inflammatory cytokines relative to wild-type mice (139). Similarly, ablating GR in endothelial cells resulted in higher mortality and cytokine production in mutant mice following LPS (140). Deleting GR in keratinocytes led to increased markers of cutaneous inflammation in the skin and in an exaggerated inflammatory response to skin irritation (141). Finally, in cardiomyocytes, GR deletion led to early cardiac hypertrophy that was associated with reduced expression of several anti-inflammatory genes, including *Zfp36* and *Lcn2* (142).

Pharmacological high-affinity GR ligands affect all innate immune cells, producing distinct functional outcomes in different cell types. For example, GC inhibit mast cell degranulation and are

tolerogenic in dendritic cells. In macrophages, high GC concentrations induce polarization toward the M2 regulatory phenotype and inhibit cytokine production, whereas low GC concentrations promote adhesion and phagocytosis. A similar diversity of responses to high and low GC doses has been described for T and B cells (reviewed in References 143 and 144). At pharmacological concentrations, GC typically inhibit the production of inflammatory cytokines and chemokines, primarily by direct transcriptional repression of cytokine genes and secondarily by upregulating genes encoding inhibitors of inflammation. A combination of expression profiling and knockout mouse studies demonstrated the importance of several GR-induced anti-inflammatory mediators, including DUSP1 (145), TSC22D3 (GILZ) (146), TNFAIP3 (147), NFKBI (148), Annexin-1 (149), and several KLF transcription factors (150). Furthermore, GC-dependent GR recruitment to GBS near these genes has been noted in several GR cistromes in both the human and mouse (for a list of putative GR targets with anti-inflammatory activities, see Reference 151).

DUSP1 dephosphorylates and, consequently, inactivates JNK and p38 kinases, leading to blunted activation of several effector transcription factors, e.g., AP1 and NFκB, ultimately inhibiting proinflammatory gene transcription (152, 153). Interestingly, the anti-inflammatory activities of DUSP1 are context specific: In the *Dusp1* global knockout, GC-mediated repression of cytokine production was impaired in macrophages (152), but not in mast cells (154). TSC22D3 (also known as GILZ), an endogenous inhibitor of NFκB and AP1, directly interacts with JUN, FOS, p65, and p52 (reviewed in Reference 155), thereby blunting the induction of many NFκB- and AP1-driven proinflammatory cytokine genes. Recently, GILZ was also shown to be involved in GC-mediated induction of the FOXP3 transcription factor, a master regulator for the development of peripheral regulatory T cells (Tregs) that confer protection against intestinal inflammation (146).

GR-mediated repression via tethering affects a large number of inflammatory mediators driven by AP1, NFκB, STAT, and C/EBP proteins (reviewed in References 58 and 156; see Section 4). As mentioned above, tethering-mediated repression by GR relies on the recruitment of additional cofactors such as GRIP1 (NCOA2/SRC2) and the adaptor protein TRIP6. GRIP1 is a GR coregulator of the p160 family (see Section 5) that serves as a platform for a large number of secondary cofactors with chromatin-modifying activities. GRIP1, as the glucocorticoid receptor–interacting protein 1 name implies, was described as a GR corepressor initially at AP1 tethering sites (61, 157) and was subsequently shown to broadly contribute to GR anti-inflammatory actions via this mechanism. Indeed, conditional deletion of *Ncoa2* (*Grip1*) in hematopoietic cells sensitized mice to LPS-mediated toxicity and cytokine storm—a phenotype typically found in mice after ablating endogenous inhibitors of inflammation (158). As expected, in macrophages derived from the conditional GRIP1 knockout mice, GC-mediated repression of a large number of proinflammatory genes—including *Tnf*; *Il1a* and *-b*; *Cxcl10* (IP10); and *Ccl2*, *-3*, and *-4*—was impaired (158). The precise molecular mechanisms of GRIP1-mediated corepression are unknown. However, GR:GRIP1 complexes appear to control, in a gene-specific manner, transcription initiation at the level of PolII recruitment and PolII pause release during early elongation (64). At elongation-controlled genes specifically, repression by GR was associated with the accumulation of the multiprotein NELF (negative elongation factor) complex that is responsible for PolII promoter–proximal stalling at the TSS (64, 159, 160).

TRIP6 is the other adaptor protein that interacts with GR at tethering GBS. It contains multimerized LIM domains that are involved in protein-protein interactions. The smallest TRIP6 isoform (nTRIP6) has been implicated in the hormone-dependent recruitment of GR to AP1 at tethering GBS by forming a ternary complex with FOS and GR (161). In the absence of GR, nTRIP6 acts as a coactivator for FOS-containing AP1 heterodimers by recruiting the mediator subunits THRAP3 (TRAP150) and MED1 (TRAP220) (162). Liganded GR interacts with nTRIP6 through the same LIM domain as does THRAP3, suggesting that GR competitively

inhibits interactions between AP1:nTRIP6 and the mediator, thereby attenuating AP1-dependent transcription (162).

Over the years, the relative contributions of GR-mediated repression versus activation to inflammation have been a matter of debate, which was driven primarily by early attempts to create new GR ligands with dissociative properties and that would function as anti-inflammatory drugs. The underlying assumption was that repression by GC is beneficial for curbing inflammation, whereas activation by GC is undesirable and leads to insulin resistance and muscle wasting. Although attractive, this overly simplistic rationale fails to reflect the system-wide complexity and diversity of GR-driven regulatory networks. Repression versus activation and, in more general terms, "beneficial" versus "adverse" effects of GC likely represent a false dichotomy that does not account for cell type–, tissue-, and species-specific variations in the repertoire of partnering proteins, ligand concentrations, and other contextual variables that affect the physiological outcome. An unexpectedly modest overlap of GBS in different cell types within the same species serves as a striking illustration of GR regulatory complexity. Similarly, time- and concentration-driven GR binding and, consequently, regulatory events observed in the mouse and human (35, 75, 150, 163) underscore the need for comprehensive genome-wide kinetic analysis of GR cistromes and transcriptomes with both natural and synthetic ligands.

8. SUMMARY AND CONCLUSIONS

Biological systems are inherently complex, and as such, their parameters, dynamics, and evolution are sensitive to the context and are constrained by historic contingencies that have led to the current context. Applied to the regulation of transcription, context is defined by the repertoire and activity of available gene regulators, signal transduction pathways, and the cellular and physiological microenvironment, whereas contingency constitutes the sequence of the past regulatory events that have produced a current steady state. Contingency influences context, as specific outcomes of past regulatory events constrain the choice and the implementation of new transcription programs. From this perspective, investigation of individual genes performed in controlled, artificial conditions can be misleading, as such an approach ignores both the context and contingency present in a multilevel systemic environment. Hormonal regulation of transcription is a perfect illustration of the following principles. (*a*) An increasing concentration of bioavailable hormone triggers genome-wide DNA binding by the receptor. (*b*) The repertoire of GBE is constrained by the preexisting chromatin landscape that was established prior to hormonal stimulation and can be further modified by the amount of active receptor and partnering factors. As the chromatin landscape is determined by lineage-specific transcription factors, these constraints profoundly influence GBE repertoire in a cell type–specific manner. (*c*) Prolonged hormonal stimulation or combination of signals can change the chromatin landscape and ultimately create new transcriptional states. Context sensitivity of the hormonal response is at the heart of the high variability of responses to seemingly similar initial stimuli between individual cells, tissues, organs, and whole organisms. Indeed, a large number of GBS identified in early studies with simple reporter constructs failed to function in vivo; others displayed extreme cell type specificity or became engaged only following a very narrow combination of stimuli. Studies in knockout mice also tend to ignore context and contingency, as such studies focus primarily on easily detectable phenotypes in response to simple monofactorial cues and usually do not account for redundancies and compensatory mechanisms. The study of complex systems requires methods that match their complexity and that simultaneously capture both steady-state and dynamic parameters at multiple levels. This framework is especially applicable to hormonal regulation that targets multiple cell types and organs, that involves numerous feedback and feed-forward regulatory loops, and that translates from the level of

tissues and organs to behavioral changes in individuals that ultimately affect populations at large. In this respect, further development of genomic and computational methods holds great promise in addressing a key challenge in modern biology: linking mechanistic and molecular studies to specific phenotypes that are determined by networks of genes and shaped by the environment.

DISCLOSURE STATEMENT

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